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(באנגלית) (English)
PROCESS FOR LARGE SCALE PURIFICATION OF ALPHA – 1 PROTEINASE INHIBITOR

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PROCESS FOR LARGE SCALE PURIFICATION OF ALPHA-1 PROTEINASE INHIBITOR

תהליך לניקוי בקנה מידה גדול של מעכב פרוטאינז אלפא-1

KAMADA/002 IL

PROCESS FOR LARGE SCALE PURIFICATION OF ALPHA-1 PROTEINASE INHIBITOR

FIELD OF THE INVENTION

The present invention relates to a process for the purification of alpha-1 proteinase inhibitor (API) from a mixture of unpurified proteins. More particularly, the present invention relates to a process for the purification of API from blood plasma or from plasma fractions to obtain pharmaceutical grade API on a commercial scale. The present invention also relates to formulations comprising the purified API and methods of using same.

BACKGROUND OF THE INVENTION

Certain human plasma proteins useful for therapeutic purposes and other applications can be obtained from outdated pooled blood donations. Recombinant production is complicated by the fact that these proteins require accurate glycosylation patterns in order to maintain their function. Therefore even with the attendant risks of viral or other contamination the only approved available source is human plasma itself.

Alpha-1 proteinase inhibitor (API) is a glycoprotein having a molecular weight of 53,000 daltons. The protein is a single polypeptide chain, to which several oligosaccharide units are covalently bound. API has a role in controlling tissue destruction by endogenous serine proteinases, and is the most prevalent serine protease inhibitor in blood plasma. Among others, API inhibits trypsin, chymotrypsin, various types of elastases, skin collagenase, renin, urokinase and proteases of polymorphonuclear lymphocytes.

API is currently used therapeutically for the treatment of pulmonary emphysema in patients who have a genetic deficiency in API. Purified API has been approved by

the FDA for replacement therapy in these patients. The normal role of API is to regulate the activity of leukocyte elastase, which breaks down foreign proteins present in the lung. When API is not present in sufficient quantities to inhibit elastase activity, the elastase breaks down lung tissue. In time, this imbalance results in chronic lung tissue damage and emphysema.

The demand for API already exceeds the availability of the current supply, and this problem may become more pronounced as research suggests additional therapeutic uses for API. The limited availability of human plasma, which is currently the source for therapeutic grade API, contributes to the short supply. In order to maximize the available supply of API, a process for purifying API from human plasma should have the highest yield possible, and alternative sources should be also considered. The purity of the API isolated form human plasma is also critical, because trace impurities can stimulate immune responses in patients who are receiving API. Therefore, more efficient means of isolation, suitable for GMP (good manufacture practice) large-scale production, are required.

Several groups have reported production of recombinant API. (For example, G. Wright et al., Biotechnology, Vol. 9, pp. 830-834 (1991); A. L. Archibald et al., Proc. Nat'l. Acad. Sci. USA., Vol.87, pp. 5178-5182 (1990)). However, at present human plasma is the only approved source of therapeutic API.

Various methods of purifying API from human plasma have been described. The majority of these methods are directed to laboratory scale isolation while others pertain to production on a commercial level. Several methods of isolation are disclosed, for example in U.S. Patent Nos. 4,379,087 and 5,610,285 .Many early methods employed ammonium sulfate precipitation from human plasma and dialysis, followed by a subsequent chromatographic step on DEAE-cellulose. However, the methods described for dialysis are not easily applicable to large-scale purification, and are lengthy, time-consuming processes likely to compromise the activity of the isolated protein.

A large-scale purification of API from human plasma was disclosed by Kress et al., Preparative Biochem., 3:541-552 (1973). The precipitate from the 80% ammonium sulfate treatment of human plasma was dialyzed and chromatographed on

DEAE-cellulose. The concentrate obtained was again dialyzed and gel filtered on SEPHADEX™ G-100. The API-containing fractions were chromatographed twice on DE-52 cellulose to give API.

Glaser et al., Preparative Biochem., 5:333-348 (1975) isolated API from Cohn Fraction IV-1. In this method, dissolved IV-1 was chromatographed on DEAE-cellulose, QAE-SEPHADEX™, concanavalin A-SEPHAROSE™, and SEPHADEX™-G-150 to give API. However, Glaser et al. achieved only a 30% overall yield.

Podiarene et al., Vopr. Med. Khim. 35:96-99 (1989) reported a single step procedure for isolation of API from human plasma using affinity chromatography with monoclonal antibodies. API activity was increased 61.1 fold with a yield of only 20%.

Burnouf et al., Vox. Sang. 52: 291-297 (1987) starting with Cohn Fractions II+III used DEAE chromatography and size exclusion chromatography to produce an API 80-90% pure (by SDS-PAGE) with a recovery of 65-70%.

Hein et al., Eur. Respir. J. 9:16s-20s (1990) presented a process that employs Cohn Fraction IV-1 as the starting material and utilized fractional precipitation with polyethylene glycol followed by anion exchange chromatography on DEAE-Sepharose™. The final product has a purity of about 60% with 45% yield.

Dubin et al., Prep..Biochem. 20: 63-70 (1990) used a two-step chromatographic purification whereby alpha-PI, C_1-inhibitor, alpha-1 antichymotrypsin, and alpha-1 trypsin inhibitor were first eluted from Blue Sepharose™ and then API was purified by gel filtration. Purity and yield data were not given.

U.S. Patent No. 4,749,783 described a method where biologically inactive proteins in a preparation were removed by affinity chromatography after a viral inactivation step. The basis of the separation between the native and denatured forms of the protein was the biological activity of the native protein towards the affinity resin and not physical differences between the native and denatured proteins.

An integrated plasma fractionation system based on polyethylene glycol (PEG)

was disclosed by Hao et al. (Proceedings of the International Workshop on Technology for Protein Separation and Improvement of Blood Plasma Fractionation, Sept. 7-9, 1977, Reston, Va). In the published method Cohn cryoprecipitate was mixed with increasing concentrations of PEG in order to obtain four different PEG fractions. The four fractions obtained were 0-4% PEG precipitate, 4-10% PEG precipitate, 10-20% PEG precipitate and 20% PEG supernatant. The 20% PEG supernatant fraction was dominated by albumin but also contained most of the API. However, this fraction also contained numerous other proteins, including all of the alpha-1-acid glycoprotein, antithrombin III, ceruloplasmin, haptoglobin, transferrin, Cl esterase inhibitor, prealbumin, retinol binding protein, transcortin, and angiotensinogen.

Several other groups have combined PEG precipitation with other purification methods in an attempt to isolate API. For instance, U.S. Patent Nos. 4,379,087; 4,439,358; 4,697,003 and 4,656,254, all employ a PEG precipitation step in processes of isolating API. However, when such methods are not accompanied with an efficient, highly reliable virus inactivation steps, PEG precipitation may be disadvantageous in that PEG will also precipitate contaminating viruses.

Along with PEG precipitation, U.S. Patent No. 4,379,087 also reports a concentration step involving phosphate buffer and DEAE SEPHAROSE™. The combined process is quite lengthy, i.e., five days. Furthermore, the final product is only 60% active and only 80% pure.

Japanese Patent No. 8-99999 describes using PEG precipitation in combination with an SP-cation exchanger. The methods described therein do not separate fully active API from inactive API. The specific activity of fully active API should be 1.88 (using an Extinction coefficient 5.3), but the product achieved by this process only shows a relative activity of 1.0. Moreover, the best yield, achieved by combining PEG precipitation and SP-cation exchange steps, was only 50%, and does not appear to be easily scaled up to a commercial production level.

U.S. Pat. No. 5,610,285 discloses a purification process which combines successive anion and cation exchange chromatography steps. The initial anion exchange chromatography step binds API to the column; however, it also binds

numerous contaminating proteins, particularly lipoproteins. Lipoproteins are plentiful in many of the materials from which API is isolated (e.g. Cohn IV paste), and so tend to occlude the column. Such occlusion requires columns of considerable size, additional dialysis/filtration steps, and at least two cation chromatography steps. Those requirements reduce efficiency and practicality of the method for large-scale processes. Further, in the '285 process all API, both inactive and active protein, bind to the anion exchange column. And when the API is eluted from that column in accordance with that method, i.e., high salt phosphate buffer, both active and inactive protein come off the column. Thus, there is no separation of the active from the inactive protein.

U.S. Patent No. 6,093,804, to one of the inventors of the present invention, discloses a method combining removal of lipoproteins from the source material, followed by subsequent anion and cation exchange steps, which result in highly purified, highly active API. However, this method proved to be efficient for small to mid-scale production of processing source material in the range of few kilograms.

As mentioned above, the demand for API exceeds available supply. Thus, there is a great need for, and it would be highly advantageous to have a process for a large-scale production of API in the range of tens of kilograms, in which quality - purity and activity-is not compromised for quantity.

SUMMARY OF THE INVENTION

The present invention relates to a process for the production of alpha-1 proteinase inhibitor (API), suitable for processing scaled-up amounts of source material in the range of tens of kilograms, and which yields a highly purified, highly active API. The present invention also relates to formulations comprising the purified API and methods of using same.

The process provided by the present invention combines removal of contaminating substances (i.e., lipids, lipoproteins and other proteins) and separation of active from inactive API by sequential chromatography steps. The improvement in

the process of the present invention over other processes is its adjustment for large-scale production. Such improvement is achieved by employing a combination of two methods for the removal of contaminating substances from an initial protein suspension, by the use of successive anion, cation and anion exchange resins with specific eluants and by meeting the GMP requirements of a large-scale production, in particular the methods of the present invention employ a minimum number of different buffers; automated preparation of solutions; use of solutions which can be kept under ambient storage conditions; and in particular avoid buffers and reagents prone to microbial contaminations. The purified API according to the present invention is at least 90%, preferably at least 95% pure (i.e 95% w/w of the total protein) and of the purified API at least 90% is active. The yield of the disclosed large-scale process is at least 50%, and typically at least 60%.

According to certain embodiments the end product of the process of the present invention is a liquid suitable for direct use. This currently preferred embodiment is advantageous to an end product in the form of a powder, which requires additional drying and subsequent reconstitution steps prior to administration.

According to one aspect, the present invention provides a process for the production of highly purified, active API in a liquid form. According to one embodiment, the present invention provides a process for purifying alpha-1 proteinase inhibitor (API) from an unpurified mixture of proteins comprising:

a. dispersing the unpurified mixture of proteins containing API in an aqueous medium;
b. removing a portion of contaminating lipids and proteins by adding a lipid removal agent and precipitating the portion of contaminating proteins from said aqueous dispersion;
c. loading the supernatant of (b) containing API on a first anion exchange resin with a buffer solution having pH and conductivity such that API is retained on the first anion exchange resin;
d. eluting an API-containing fraction from said first anion exchange resin with the same type of buffer having adjusted pH and conductivity;
e. loading the API-containing fraction of step (d) on a cation exchange

resin in said same type of buffer having appropriate pH and conductivity such that API is not retained on the cation exchange resin;

f. collecting the flow-through of step (e) that contains API;

g. loading the API-containing fraction of step (f) on a second anion exchange resin with said same type of buffer having appropriate pH and conductivity such that API binds to the second anion exchange resin;

h. eluting API from said second anion exchange resin with said same type of buffer having adjusted pH and conductivity to obtain a solution containing purified, active API.

According to one embodiment, the process of the present invention provides purified API comprising at least 90%, preferably at least 95% API out of the total protein, wherein at least 90%, preferably 95% of the pure API is active.

Throughout the process of the present invention only one type of buffer is used, with adjustment of pH and conductivity as required throughout the various process steps. According to one embodiment, the buffer is any suitable organic acid/salt combination that provides acceptable buffer capacity in ranges of pH required throughout the process of the present invention. According to preferred embodiments the process uses a buffer other than citrate-based buffer. According to yet another embodiment, the buffer anion is acetate. According to a further embodiment, the buffer solution is sodium acetate.

According to one embodiment, the process of the present invention further comprises viral removal and/or viral inactivation steps. Methods for viral removal and inactivation are known in the art. According to one embodiment, the viral removal step comprises filtration. Both enveloped and non-enveloped viruses are removed by filtration, preferably by nanofiltration, or any other filtration methods as are known in the art. According to one embodiment, a virus removal step is performed after the cation exchange chromatography. The cation exchange flow-through solution containing API may be concentrated, and then nano-filtered.

According to one embodiment, the method of viral inactivation employed by the present invention comprises a solvent/detergent (S/D) treatment. The viral inactivation step is preferably performed prior to loading the solution on the second

anion exchange resin. According to one embodiment, the detergent used is polysorbate and the solvent is Tri-n-Butyl-Phosphate (TnBP). According to another embodiment, the polysorbate is polysorbate 80. According to one embodiment Polysorbate 80 may be added at from about 0.9% to about 1.3% weight per weight of the resulting mixture and TnBP from about 0.2% to about 0.4% weight per weight of the resulting mixture.

According to one embodiment, the unpurified mixture of proteins comprising API is dispersed in water, and the pH of the dispersion is adjusted to a pH range of from about 9.0 to about 9.5. The pH adjustment stabilizes the API and promotes the dissolution of the API in the dispersion, thereby increasing the production yield. The unpurified mixture of proteins may be of any source, provided it contains a substantial amount of API. According to one embodiment, the API-containing protein mixture is selected from plasma, particularly from Cohn fractions IV. According to one another embodiment, the API-containing protein mixture is Cohn fraction IV-I.

According to one embodiment, the lipid removal agent utilized for the removal of lipids and lipoproteins is silicon dioxide (Aerosil™). The contaminating proteins are precipitated with polyalkylene glycol. According to one embodiment, the polyalkylene glycol is polyethylene glycol. According to yet another embodiment, the pH of the dispersion is reduced before the addition of the polyalkylene glycol. The pH reduction improves the precipitation, and the lipid removal agents and the precipitate are removed by centrifugation. The supernatant from this step is in a pH range suitable for the first anion exchange chromatography. According to one embodiment, the pH is reduced to a pH range of from about 5.0 to about 6.5. To further prepare the supernatant for loading on the anion exchange resin its conductivity is adjusted to from about 0.5 to about 3.5 mS/cm.

According to one embodiment, the first and the second anion exchange resin is a DEAE-Sepharose resin and the cation exchange resin is Carboxymethyl-Sepharose resin. The chromatography sequential steps are performed with a single type of buffer throughout the process. However, individual sequential steps are performed under different pH and conductivity conditions by adjustment of theses parameters in the buffer. It has been previously shown that the separation of active from inactive API

can be achieved by ion exchange chromatography. The cation exchange resin is used to further purify the API-containing fraction from substances that bind to the cation exchange resin, while the API passes through the resin. According to one embodiment, the pH of the API-containing fraction is adjusted to between 5.3 and 5.6 and the conductivity to from about 0.8 to about 1.1 mS/cm before loading the API-containing fraction on the cation exchange fraction.

The present invention further comprises methods for separating active API from other contaminating substances, including solvent/detergent compounds used for viral inactivation as described herein above. According to one embodiment, this separation is advantageously achieved by the second anion exchange chromatography. According to one embodiment, before loading on the second anion exchange resin the pH of the API-containing fraction is adjusted to about 7.0 and the conductivity to about 3.0 mS/cm.

According to another aspect, the present invention relates to formulations comprising a purified active API produced by the process of the present invention.

According to one embodiment, the solution containing purified, active alpha-1 proteinase inhibitor is concentrated and then the buffer is exchanged to phosphate buffer and the protein concentration is adjusted as required. The resulted solution is designated herein as "drug substance". The drug substance is filter-sterilized to obtain a final formulated product, designated herein as "drug product". The drug product of the present invention is currently used in clinical trials.

According to one embodiment, the protein concentration is adjusted to the range of 20-40 mg/ml. According to one embodiment, the end product of the process of the present invention is a ready-to-use liquid.

The drug product of the present invention is highly stable. According to one embodiment the drug product is stable, with no reduction in API activity, when kept at a temperature of between 20°C to 25°C for at least 6 months. According to another embodiment, the drug product is stable with no reduction in activity when kept at between 2°C to 8°C for at least 12 month, and up to 36 months.

According to one embodiment, the present invention provides a method for treating a subject in need thereof comprising administering a pharmaceutically acceptable amount of API produced by the process of the present invention. According to one embodiment, the method is used for treating pulmonary emphysema.

The present invention is explained in greater details in the description, figures, and claims below.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 describes the protein profile on a native Tris-Glycine gradient gel during the API production process. Fig. 1A: Coomassie blue stained gel. Fig.1B: Ponceau-S stained gel. Fig. 1C: Immunoblot with Goat anti-API, HRP-conjugate antibodies. Lanes of the gels are as follows: 1, 2 –Final drug product; 3, 4 – In house API standard; 5 – Albumin; 6 – Transferrin; Anti-D (IgG); 8, 9, 10 – Polymeric fraction of API.

FIG. 2 describes the protein profile on a 4%-12%gradient SDS-PAGE during the API production process. Fig. 2A: Coomassie blue stained gel. Fig.2B: Ponceau-S stained gel. Fig. 2C: Immunoblot with Goat anti-API, HRP-conjugate antibodies. Lanes of the gels are as follows: 1 - Sample buffer; 2, 3 - dispersion before the addition of Aerosil; 4, 5: Dispersion before the addition of PEG; 6,7: eluate after first anion exchange chromatography; 8, 9: ultrafiltration after cation exchange chromatography; 10: eluate after second anion exchange chromatography; 11: end product of the process (drug substance); 12, 13: formulated API (drug product); 14, 15 – commercial and in-house molecular weight standards, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a process for the production of highly pure, highly active alpha-1 proteinase inhibitor on commercial scale. A particular advantage

of the process provided by the present invention is its efficacy in processing source material in the range of tens of kilograms, without compromising purity and activity thereof, being at least 95% and at least 90%, respectively.

According to one embodiment, the present invention provides a process for purifying API from an unpurified mixture of proteins comprising:

a. dispersing the unpurified mixture of proteins containing API in an aqueous medium;

b. removing a portion of contaminating lipids and proteins by adding a lipid removal agent and precipitating the portion of contaminating proteins from said aqueous dispersion;

c. loading the supernatant of (b) containing API on a first anion exchange resin with a buffer solution having pH and conductivity such that API is retained on the first anion exchange resin;

d. eluting an API-containing fraction from said first anion exchange resin with the same type of buffer having adjusted pH and conductivity;

e. loading the API-containing fraction of step (d) on a cation exchange resin in said same type of buffer having appropriate pH and conductivity such that API is not retained on the cation exchange resin;

f. collecting the flow-through of step (e) that contains API;

g. loading the API-containing fraction of step (f) on a second anion exchange resin with said same type of buffer having appropriate pH and conductivity such that API binds to the second anion exchange resin;

h. eluting API from said second anion exchange resin with said same type of buffer having adjusted pH and conductivity to obtain a solution containing purified, active API.

This process provides API fractions of at least about 90% API out of the total protein; often providing fractions of greater than about 95% pure API; and can achieve fractions of 99% pure API. Of the API, at least 90% is active. At least 95% active API is also achieved. Activity of API is measured by trypsin inhibition as exemplified herein below.

The anion exchange chromatography is the principle stage in which active API

is separated from inactive API, as disclosed by one of the inventors of the present invention in U.S. Patent No. 6,093,804. However, the method described in said US patent, adjusted to a small to mini-scale production, utilizes various buffer types to achieve such separation (loading the anion exchange with non-citrate buffer and eluting an API-containing fraction with citrate-based buffer). The present invention provides a process which meets the requirements of large-scale production, one of them being the use of minimal number of different solutions. The present invention therefore discloses the use of one type buffer in all chromatography steps, while adjusting the pH and conductivity of the buffer as required throughout the various process steps. According to one embodiment, the buffer is any suitable organic acid/salt combination that provides acceptable buffer capacity in ranges of pH required throughout the process of the present invention. According to preferred embodiments the process uses a buffer other than citrate based buffer. According to yet another embodiment, the anion buffer is acetate. According to a further embodiment, the buffer solution is sodium acetate.

According to one embodiment, the process of the present invention further comprises viral removal and/or viral inactivation steps. Viral reduction can be accomplished by several processes, including nanofiltration; solvent/detergent treatment; iodine inactivation, e.g., treatment with an iodinated ion exchange matrix material such as iodinated SEPHADEX™ (as disclosed in PCT applications WO 97/48422 and WO 97/48482); treatment with Pathogen Inactivating Compounds; heat inactivation; gamma irradiation; or any other suitable virucidal process.

According to one embodiment, the viral removal step comprises filtration. Both enveloped and non-enveloped viruses are removed by filtration, preferably by nanofiltration, or any other filtration methods known in the art. According to one embodiment, a virus removal step is performed after the cation exchange chromatography. According to one embodiment, the cation exchange flow-through solution containing API may be concentrated by ultrafiltration. Prior to nanofiltration, the pH of the concentrated retentate may be adjusted to from about 6.8 to about 7.7, and its conductivity to from about 2.5 to about 3.5 mS/cm. The filtrate ("nanofiltrate") is collected for the subsequent step of viral inactivation.

According to one embodiment, the method of viral inactivation employed by the present invention comprises a solvent/detergent (S/D) treatment. This step is preferably taken prior to loading the solution on the second anion exchange resin. According to one embodiment, the detergent used is a non-ionic detergent such as polysorbate and the solvent is TnBP. According to another embodiment, the polysorbate is polysorbate 80. According to one embodiment Polysorbate 80 may be added at from about 0.9% to about 1.3% weight per weight of the resulting mixture and TnBP from about 0.2% to about 0.4% weight per weight of the resulting mixture. According to one embodiment, S/D viral inactivation is performed in a pH range of between about 7.0 and 8.0 and conductivity range of between about 2.0 and 4.0 mS/cm.

The unpurified mixture of proteins from which the API is collected is preferably Cohn Fraction IV-1 paste, but can include other Cohn Fractions, separately or in combination, human blood plasma, plasma fractions, or any protein preparation containing API. For instance, the present process is applicable to purification of recombinant human API from the milk of transgenic animals. (When milk is used as starting material, an ammonium sulfate or sodium chloride precipitation step is first employed to separate API from caseins, and the precipitate is taken through the present purification process.) According to one embodiment, the unpurified mixture of proteins comprising API is dispersed in an aqueous medium, preferably water, at a ratio of between about 20 to about 35 liter per about 1 kg of Cohn Fraction IV-1 paste. The pH of the dispersion is adjusted to a pH range of from about 9.0 to about 9.5. The pH adjustment stabilizes the API and promotes the dissolution of the API in the dispersion, thereby increasing the production yield. Dispersion may take place at elevated temperature, for further increase in API solubility. According to one embodiment, dispersion is performed at a temperature of between 35°C and 40°C.

A particular advantage of the present invention is the ready elimination of contaminants or by-products that otherwise compromise the efficiency of API purification processes. Cohn Fraction IV-1 preparations in particular contain a significant amount of the lipoprotein Apo A-1, which has the effect of inhibiting column flow and capacity during purification, and other proteins such as albumin and transferrin. Removing a portion of such contaminant is performed according to the

present invention by two sequential steps: removing contaminating lipids and lipoproteins by lipid removal agent and precipitating a portion of contaminating protein from the API-containing aqueous dispersion.

According to one embodiment, the lipid removal agent is silicon dioxide (Aerosil™). The Aerosil™ is added at a ratio of 1:10 to 1:14 Aerosil™: Kg paste. This step is performed at a high pH of about 9.0, and the resulting mixture is stirred for about 60-120 min. at a temperature of between 35oC and 40oC. According to one embodiment, polyalkylene glycol is used for precipitating the portion of contaminating proteins, for example polyethylene glycol (PEG) or polypropylene glycol (PPG). Other alcohols known to those skilled in the art to have similar properties may be used. According to one embodiment, polyethylene glycol is used. According to yet another embodiment, the PEG used in the process of the present invention has a molecular weight of between 2,000 and 10,000, preferably has a molecular weight of between 3,500 and 4,500. The PEG added to the solution is at least about 2% weight per volume of the mixture formed. According to one embodiment, the PEG added is about 3% to 15%. According to another embodiment, PEG is added at between 10 to 12% weight per volume of the resulting mixture. Before the addition of the polyalkylene glycol the temperature of the mixture is adjusted to room temperature (at the range of from about 20oC to 25oC) and the pH of the dispersion is reduced. The pH reduction improves the precipitation and the supernatant from this step is in a pH range suitable for the first anion exchange chromatography. According to one embodiment, the pH is reduced to a pH range from about 5.0 to about 6.5 by the addition of, for example, acetic acid. In addition, a salt such as sodium chloride or the like may be added to the aqueous mixture in an amount sufficient to achieve a conductivity of from about 0.5 to about 3.5, to further prepare the supernatant for loading on the anion exchange resin. The removal of contaminating proteins, without loss of API, enables a significant reduction in equipment scale, e.g., column size.

The precipitate that forms can be separated by conventional means such as centrifugation and discarded, and the supernatant is ready for further purification as described hereinbelow. All the above-described steps for removing contaminating substances are performed in one container, which is highly advantageous for a

commercial, large-scale production process.

The above-described supernatant is then loaded on an anion exchange resin such as DEAE-Sephadex, QAE-Sephadex, DEAE-Sephacel, DEAE-cellulose, DEAE-Sepharose or the like. According to one embodiment, the anion exchange resin is DEAE-Sepharose. Variety of conditions may be used in this particular step. For best results the anion exchange medium is placed in a chromatographic column and the API eluted therefrom. According to one embodiment, the anion exchange resin is first equilibrated with step-wise buffer application, starting with a solution of pH about 3.5-4.5 and a conductivity of from about 8.0 to 12.0 mS/cm, and then with a solution of pH about 5.5 –6.5 and a conductivity of from about 2.5 to about 3.5 mS/cm. Next, the above-described supernatant is loaded on the first anion exchange resin. These conditions of pH and conductivity allow the retention of API on the column. The anion exchange medium is washed. The conductivity of the washing buffer (at a pH of about 5.5-6.5) is increased from about 2.0-2.2 to about 2.8-3.2 during the washing. This increase provides suitable conditions such that the column is loaded in its full capacity, and yet no API is discarded in the flowthrough, to give maximal API yield.

The API is then eluted from the column. According to one embodiment, elution is performed with a buffer solution at a pH of about 5.5 to 6.5 and conductivity of from about 9.0 to about 11 mS/cm.

Following separation of a solution containing API from an ion exchange resin, the solution is treated to reduce its water content and change the ionic composition by conventional means such as by diafiltration, ultrafiltration, lyophilization, etc., or combinations thereof.

According to one embodiment, the API-containing effluent obtained after the first anion exchange chromatography is concentrated by ultrafiltration. The retentate is then diafiltered against pure water to reach conductivity within the range of from about 3.5 to about 4.5 mS/cm.

To further purify the API-containing solution obtained after the first anion exchange chromatography the solution is loaded on a cation exchange resin with the same type of buffer used for the anion-exchange step, having appropriate pH and

conductivity such to allow the API to be washed with the buffer flowthrough, while contaminating substances are retained on the action exchange resin.

According to one embodiment, the cation exchange resin is Carboxymethyl-Sepharose resin, placed in chromatography column. The cation exchange resin is first equilibrated with step - wise buffer application, starting with a solution of pH about 3.5-4.5 and a conductivity of from about 8.0 to 12.0 mS/cm, and then with a solution of pH about 5.5 – 6.5 and a conductivity of from about 0.8 to about 1.1 mS/cm. The API-containing fraction is loaded on the column with the same buffer as in the second equilibration step (pH about 5.5 – 6.5 and a conductivity of from about 0.8 to about 1.1 mS/cm) and the flowthrough is collected.

Again, as disclosed herein above, the API-containing solution obtained after the cation exchange chromatography can be treated to reduce its water content. According to one embodiment, the solution is concentrated by ultrafiltration.

As disclosed herein above, the anion-exchange chromatography is used principally to separate active API from inactive API. The present invention further comprises methods for separating active API from other contaminating substances, including solvent/detergent compounds used for viral inactivation as described herein above.

According to one embodiment, such separation is achieved by the second anion exchange chromatography. The present invention shows that advantageously, contaminating substances, particularly non-ionic detergents and solvents commonly used for viral inactivation, does not retain in the DEAE-Sepharose anion exchange resin under the conditions of the present invention as detailed herein below. The API eluted from the second anion exchange chromatography step is therefore not only highly active but also highly pure. The anion exchange resin is first equilibrated with step-wise buffer application, starting with a solution of pH about 3.5-4.5 and a conductivity of from about 8.0 to 12.0 mS/cm, and then with a solution of pH about 5.5 –6.5 and a conductivity of from about 2.5 to about 3.5 mS/cm. Next, the API-containing fraction, typically, after viral inactivation treatment, is loaded on the second anion exchange resin. At this stage, the pH of the loading buffer may be elevated, and washing can be performed in one step, as the solution is already purified

from the majority of contaminating proteins. According to one embodiment, the pH of the loading buffer is from about 7.0 to about 8.0 and the conductivity from about 2.0 to about 4.0 mS/cm. The pH of the washing buffer is about 5.5-6.5, and the conductivity about 2.8-3.2.

The API is then eluted from the column. According to one embodiment, elution is performed with a buffer solution at a pH of about 5.5 to 6.5 and conductivity of from about 11 to about 13 mS/cm.

The solution containing active, purified API obtained after the second anion exchange chromatography can be formulated into pharmaceutical preparations for therapeutic, diagnostic, or other uses. To prepare them for intravenous administration the compositions are dissolved usually in water containing physiologically compatible substances such as sodium chloride, glycine, and the like and having a buffered pH compatible with physiological conditions.

According to one embodiment, the ionic composition of the solution containing active, purified API is changed to contain the physiologically compatible phosphate ion by diafiltration against sodium phosphate buffer, at a physiological pH of about 7.0. The resulted API-containing solution is also designated hereon as "drug substance". The resulted solution is then concentrated and filter-sterilized to obtain a liquid solution suitable for intravenous administration. This final solution is designated herein as "drug product".

The drug product of the present invention is highly stable. According to one embodiment the drug product is stable, with no reduction in API activity, when kept at a temperature of between 20°C to 25°C for at least 6 months. According to another embodiment, the drug product is stable with no reduction in activity when kept at between 2°C to 8°C for at least 12 month, and up to 36 months.

The filter-sterilized API-containing solution (drug product) can be used directly, and also can be incorporated into pharmaceutical preparations which may be used for therapeutic purposes. The term "pharmaceutical preparation" is intended in a broader sense herein to include preparations containing a protein composition in accordance with this invention used not only for therapeutic purposes, but also for reagent or

diagnostic purposes as known in the art or for tissue culture. The pharmaceutical preparation intended for therapeutic use should contain a therapeutic amount of API, i.e., that amount necessary for preventative or curative health measures. If the pharmaceutical preparation is to be employed as a reagent or diagnostic, then it should contain reagent or diagnostic amounts of API.

The drug product of the present invention is highly pure, containing at least 90% API out of the total proteins, typically containing 95% to 99% API, of which at least 90% is active. Compared to other plasma-derived approved products, the API-solution obtained by the methods of the present invention does not contain any imurities such as albumin, sucrose or manitol. The API product of the present invention is also non-toxic, and it was approved by the FDA for use in phase I clinical trials.

According to one embodiment, the present invention provides a method for treating a patient in need thereof comprising administering a therapeutically effective amount of API produced by the process of the present invention. According to one embodiment, the method is used for treating pulmonary emphysema.

The principles of the invention, purifying active alpha-1 proteinase inhibitor from an unpurified mixture of proteins on a commercial scale, may be better understood with reference to the non-limiting examples below.

EXAMPLES

API is estimated by its trypsin inhibitory capacity, using a chromogenic substrate for trypsin. Hydrolysis of N-succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (SA_3pNA) by trypsin causes an increase in absorption at 405 nm. This increase is continuously monitored usually at 37°C. Comparisons of the linear changes of absorbance with time in the presence and absence of sample (API) are made. The amount of inhibitor is then calculated based on the known molecular weights of trypsin and API, on the known 1:1 stoichiometry, and on the known amount of trypsin used.

Example 1: Pretreatment of the source material

In a preferred embodiment, the starting material is Cohn Fraction IV-1 paste, which is obtained by the Cohn-Oncley fractionation technique, well known to those of skill in the art. The preparation of an aqueous solution from the Fraction IV -1 paste is described below.

The IV-1 paste is dissolved in about 35 volumes of water-for-injection grade water, (IV-1 paste weight in kg times 35). The amount of starting paste is 75-87 Kg per run, added to a jacketed stainless steel tank in portion. The pH of the mixture is adjusted to 9.2 immediately after mixing of the first portion, and it was further adjusted by 0.5N NaOH until all the paste and water are added. The solution is mixed for approximately 10 minutes.

Fraction IV-I, like other plasma fractions, contains various proteins, such as lipoproteins, immunoglobulins, globulin, metalloproteins, etc. These proteins must be separated from the API, but some will also bind to an ion exchange resin and thereby interfere with the purification of API. Before adding the solution to an anion exchange resin, therefore, a portion of these contaminating proteins is preferably removed. According to the present invention, removing such contaminating fraction is performed by two steps.

Removing of lipids and lipoproteins

To the dispersion obtained above a lipid-removing agent, Aerosil™ (silicon dioxide) was added at 78-82 g/Kg paste. After the addition of Aerosil, the pH of the resulting mixture was adjusted to 8.8 with NaOH 0.5N. The mixture was incubated for 90 min. at 38°C and a stirring rate of 870-1450 rpm. After 90 min. the dispersion was cooled to 22.5°C and the pH was adjusted to 6.1.

Precipitation of contaminating proteins

To the cooled dispersion obtained as above Polyethylene glycol (PEG) of mean molecular weight of 4,000 kd was added at 10.5-11.5% weight per volume of the resulted mixture while stirring at 2660-2900 rpm. After PEG dissolution the pH of the

dispersion was adjusted to 6.0 with 2% acetic acid. Conductivity was adjusted with solid NaCl to 3.0 mS. A precipitate of contaminating proteins and viruses, including prion proteins, was formed. This precipitate was removed by continues centrifugation (Self-desludging centrifuge, model CSA19-06-476, Westfalia) at a centrifugation rate of 300-450 liter/hr. The sediment obtained by the centrifugation was discarded; the supernatant was further filtered using 1 μm (nominal) cellulose fiber depth filter and a pressure of $\leq$ 25 psi.

Example 2: First anion exchange chromatography

The resin used for the first anion exchange chromatography was DEAE-Sepharose fast Flow, packed in a stainless steel 316-L column (CF 1000/150 SS CHROMAFLOW, Pharmacia), having a volume of 117 liter.

The DEAE-Sepharose resin was first equilibrated step-wise with sodium acetate buffer as follows:

a) pH 4.0, conductivity 10.0 mS/cm
b) pH 6.0, conductivity 3.0 mS/cm

Flow rate: 7-20 liter/min.

After the procedure described in example 1 the filtered supernatant obtained was already at the suitable pH and conductivity conditions of pH 6.0 and 3.0 mS/cm. It was therefore directly loaded on the equilibrated DEAE-Sepharose column, at a flow rate of 12-14 liter/min.

In such large-scale production, it is critical to adjust the washing conditions such that the column will be loaded at its maximal capacity, and yet that no API would leak out in the flow through. This was achieved by a two-step column wash, both with sodium acetate buffer at the following conditions: first wash - pH 6.0, conductivity 2.1 mS/cm; second wash: pH 6.0, conductivity 3.0 mS/cm. The API is retained on the column, and other proteins, for example albumin and transferrin are washed out.

Elution of the API from the column was performed with a sodium acetate buffer having a pH of 6.2 and conductivity of 10.0 mS/cm. The pressure on the column was $\leq$ 35 psi and the flow rate 12-14 liter/min. TheAPI-containing fraction was then treated to adjust it water contact and ion composition as described in Example 3 below.

Example 3: Adjustment of water content and ion composition

The API- containing effluent was concentrated to 100 kg (total weight) by ultrafiltration with polysulfone membrane with a nominal cut off of 10 kD and total membrane area of 12.2 m^2 (UFP-10C-65) in a Hollow Fiber cartridge (Amersham Biosciences). The retentate pressure was 15-20 psi and the flow rate 20-40 liter/min. The filtrate is then discarded and the retentate was diaflitered against water-for-injection (WFI) grade water. The retentate pressure was 15-20 psi at a flow rate of 20-40 liter/min., and the diafiltration was continued until the solution reached an OD_{280} of 2.5 and conductivity of 4.0 mS/min.

Example 4: Cation exchange chromatography

To further purify the API-containing fraction obtained after the procedure of example 3 from remaining contaminating substance, the solution was subjected to cation exchange chromatography using the same sodium acetate buffer under conditions which allow the retention of only the contaminating substances of the cation resin, while the API was washed out with the column flowthrough.

The cation exchange resin used was CM-Sepharose fast Flow, packed in a stainless steel 316-L column (CF 1000/150 SS CHROMAFLOW, Pharmacia), having a volume of 117 liter.

The resin was equilibrated with sodium acetate buffer under the following conditions:

a) pH 4.0, conductivity 10.0 mS;

b) pH 5.35, conductivity 0.95 mS/cm

Flow rate: 7-22 liter/min.

Before loading the API-containing fraction on the column, the conductivity of the solution was adjusted with NaCl to 0.95 mS/cm and the pH was adjusted with 2%acetic acid to 5.35. The protein concentration of the solution was 0.5%, and total protein load was $\leq$ 4 Kg. Flow rate was 18-22 liter/min at a pressure of 29 $\leq$ psi. The column flow through containing the API was collected. The pH was adjusted to 6.75 with 0.15 M NaOH and the conductivity to 3.0 mS/cm with solid NaCl. The resulting solution was ultrafiltered using polysulfone membrane with a nominal cut off of 10 Kd and total membrane area of 12.2 m^2 (UFP-10C-65) in a Hollow Fiber cartridge (Amersham Biosciences). The retentate was collected and the effluent discarded.

Example 5: Viral inactivation

In a preferred embodiment of the invention, the API-containing fraction obtained after the action exchange chromatography is subjected to viral removal and inactivation.

According to the preferred embodiment, viral removal was performed by nanofiltration. The API-containing solution was subjected to pre-filtration through polysulfone membrane with a pore size of 0.1 + 0.2 μm, nominal surface area of 0.6 m^2 (5441358 K-1 SS, Sartorius), at a pressure of $\leq$30 psi. The retentate was collected in 600 liters stainless steel 316-L jacketed and pressurized container equipped with a marine type stirrer. The retentate was diluted with WFI to protein concentration of 6.0-8.5 mg/ml, and pH and conductivity was re-adjusted to 7.25 with 0.15 M NaOH/2% acetic acid and 3.0 with solid NaCl, respectively. The temperature of the container was kept at 22.5°C.

Nanofiltration was performed with Planova 15N filter (15N1-000;Asahi Kasei Corporation) having a nominal surface are of 1.0 m^2. The operating pressure obtained by N_2 was 18.8 psi. Volume transferred per m^2 of filter was more than 250 liters.

Further to virus removal, the API-containing solution was subjected to viral

inactivation using the solvent/detergent method. Polysorbate 80 was added to 0.95-1.25% w/w final concentration and TnBp to a final concentration of 1.28-0.33% v/w. The mixture was stirred for about 4.5-5.5 hrs. at 30-50 rpm.

Example 6: Second anion exchange chromatography

Second anion exchange chromatography is employed to further purify the API-containing solution obtained after viral inactivation from the employed solvent and detergent. As during the steps taken after the first anion exchange chromatography some of the active API may have become inactive, inactive AP is also removed. Thus, the resulting solution after this step contains highly purified, active API.

The resin used for the second anion exchange chromatography was DEAE-Sepharose Fast Flow, packed in a stainless steel 316-L column (BPSS 800/150 SS, Pharmacia), having a volume of 75 liter.

The DEAE-Sepharose resin was first equilibrated step-wise with sodium acetate buffer as follows:

a) pH 4.0, conductivity 10.0 mS/cm
b) pH 6.0, conductivity 3.0 mS/cm

As the API-containing fraction was now partially purified, a faster flow rate of 18-22 liter/min. was employed. Of the same reason, the pH of this fraction could be elevated to 7.5 and the column could be washed with one set of buffer conditions. The conductivity of the loaded solution was 3.0 mS/cm, and the total protein load about 2.5 Kg. The pH of the sodium acetate washing buffer was 6.0 and its conductivity 3.0 mS/cm. Total wash volume was 1125-1200 liter at a flow rate of 18-22 liter/min.

Elution of the API from the column was performed with a sodium acetate buffer having a pH of 6.0 and conductivity of 12.0 mS/cm. The eluate of the second anion exchange column contained 96% pure API, of which more than 90% were active.

Example 7: Purity of API-containing solution through the process

Samples from different steps of the process were analyzed by techniques of protein separation and detection. Fig. 1 describes the protein profile on native Tris-Glycine 8% to 16% gradient gels. Non-denatured samples were loaded in a sample buffer (Invitrogen ™ LC2673) on the gels in the amounts detailed in table 1. Polymeric forms of API were derived from a purified API (pilot No. 6110003, Kamada) stored at 35°C for 6 months that was subjected to gel permeation chromatography using Sephacryl 200HR column. Three fractions (lanes 8-10) were collected for analysis on the native gels. The gels were run in a Tris-Glycine running buffer (10 x Invitrogen ™ LC2672). The gels were stained by Commassie blue (Fig. 1A), Ponaceau-S (Fig.1B) or blotted onto a nitrocellulose membrane (BioRad Trans Blot). Immunobloting was then performed using Gost ant-API, HRP-conjugated (ICN/Cappel, 55236), used at a dilution of 1:400 (Fig. 1C).

Table 1: Samples loaded onto the native gels

Lane(s)	Sample	Protein loaded (µg/lane)	
		A	B
1,2	Final drug product	2.5/5	1/2
3,4	In-house API standard	2.5/5	1/2
5	Albumin	3.5	1.4
6	Transferrin	3.5	1.4
7	Anti-D (IgG)	3.5	1.4
8	Polymeric fraction of API	3.6	1.44
9	Polymeric fraction of API	3.6	1.49
10	Polymeric fraction of API	3.6	1.32

Samples from other steps were separated by 4-12% SDS-PAGE. Gels were stained with Commassie Blue (Fig. 2A), Ponceau-S staining (Fig. 2B) or immunoblotted with Goat anti-API, HRP-conjugate antibodies. Samples are detailed in table 2.

Table 2: Samples loaded onto the SDS-PAGE gels

Lane(s)	Sample	Protein loaded (μg/lane)	
		A	B
1	Sample buffer		
2, 3	Pre-Aerosil	15	3
4, 5	Pre-PEG	15	3
6, 7	First DEAE eluate	4.0	0.8
8, 9	Ultrafiltration after cation exchange	2.0	0.4
10	Second DEAE eluate	2.0	0.4
11	Drug substance	2.0	0.4
12, 13	Drug product	2.0	0.4
14	MW size marker- commercial	10μl per lane	
15	MW in-house marker	4.6	2.3

Figures 1 and 2 clearly show that all contaminating proteins are removed during the process of the present invention. The final drug substance shows only one sharp and clean band.

Example 8: Preparation of API drug product

After the second anion exchange chromatography described in Example 6, the fraction containing the active, pure API was concentrated by ultrafiltration (UFP-10C-65: polysulfone membrane with nominal cut off of 10 kd and total nominal membrane area of 12.2 m^2). The filtrate was discarded and the retentate, concentrated to 60 kg was further subjected to diafiltration. Diafiltration was performed to replace the acetate ion by physiologically acceptable ion, specifically phosphate ion. The diafiltration conditions were filtration against sodium phosphate buffer, 18-22 nM in 0.6-0.8% NaCl, pH 7.0, retentate pressure of 15-20 psi. Final pH of the retentate was a physiological pH of 7.0. After second ultrafiltration, the protein concentration was brought to 22-24 mg/ml. The preparation was filter sterilized using two polysulfone filters of 0.45 and 0.2 μm (Sartorius, 5101507H9-B) in a serial placement. Filtration was performed under a pressure of $\leq$ 25 psi and the sterile filtrate was collected in pre

steam-sterilized container. The final, ready for use API- drug substance comprised 22-24 mg/ml protein at a pH of 7.0 ± 0.1, NaCl at a concentration of 6-8 mg/ml and phosphate concentration of 18-22 mM.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed chemical structures and functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A process for purifying alpha-1 proteinase inhibitor (API) from an unpurified mixture of proteins comprising:
   a. dispersing the unpurified mixture of proteins containing API in an aqueous medium;
   b. removing a portion of contaminating lipids and proteins by adding a lipid removal agent and precipitating the portion of contaminating proteins from said aqueous dispersion;
   c. loading the supernatant of (b) containing API on a first anion exchange resin with a buffer solution having pH and conductivity such that API is retained on the first anion exchange resin;
   d. eluting an API-containing fraction from said first anion exchange resin with the same type of buffer having adjusted pH and conductivity;
   e. loading the API-containing fraction of step (d) on a cation exchange resin in said same type of buffer having appropriate pH and conductivity such that API is not retained on the cation exchange resin;
   f. collecting the flow-through of step (e) that contains API;
   g. loading the API-containing fraction of step (f) on a second anion exchange resin with said same type of buffer having appropriate pH and conductivity such that API binds to the second anion exchange resin;
   h. eluting API from said second anion exchange resin with said same type of buffer having adjusted pH and conductivity to obtain a solution containing purified, active API.
2. The process of claim 1, wherein the API obtained comprises at least 90% active API out of the total API recovered.
3. The process of claim 2, wherein the API obtained comprises at least 95% active API out the total API recovered.
4. The process of claim 1, wherein the API obtained comprises at least 90% API out of the total protein recovered.
5. The process of claim 4, wherein the API obtained comprises at least 95% API out of the total protein recovered.
6. The process of claim 1, wherein the buffer solution is other then citrate based

buffer.

7. The process of claim1, wherein the buffer solution is acetate-based buffer.
8. The process of claim 1 further comprises a viral inactivation step.
9. The process of claim 8 wherein the viral inactivation step comprises adding a solvent and a detergent to the API of step (f) collected from the cation exchange resin.
10. The process of claim 9 wherein the detergent is a non-ionic detergent.
11. The process of claim 1, further comprising a viral removal step.
12. The process of claim 11, wherein the viral removing step comprises nanofiltration.
13. The process of claim 1, wherein the unpurified mixture of proteins is selected from the group consisting of Cohn Fractions, human blood plasma and plasma fractions.
14. The process of claim 13 wherein the unpurified mixture of proteins is Cohn fraction IV.
15. The process of claim 1 wherein the lipid removing agent is silicon dioxide.
16. The process of claim 1 wherein the portion of contaminating lipids and proteins is precipitated by polyalkylene glycol.
17. The process of claim 16, wherein the polyalkylene glycol is polyethylene glycol.
18. The process of any one of claims 16-17 wherein precipitation is performed at a pH from about 5.0 to about 6.5.
19. The process of claim 1, wherein the first and the second anion exchange resin is a DEAE-Sepharose resin.
20. The process of claim1 wherein the cation exchange resin is Carboxymethyl-Sepharose resin.
21. The process of claim 1, wherein the pH of the buffer solution is at a pH of between 5.5 and 6.5 for the elution of the API from the first and the second anion exchange resin.
22. A pharmaceutical composition comprising as an active ingredient a purified active API produced by the process of claim 1.
23. The pharmaceutical composition of claim 22 wherein the active API is further filter-sterilized.

24. The pharmaceutical composition of claim 23 wherein the pH of the composition is in the range of 6.5-7.5.

25. The pharmaceutical composition of claim 24 wherein the protein concentration is between about 20 mg/ml to about 40 mg/ml.

26. Use of purified API according to the process of any one of claims 1-22 for preparing a medicament substantially as shown in the specification.

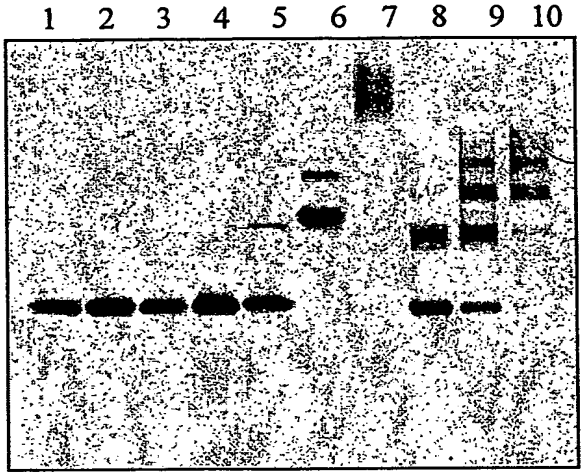
27. The use of claim 26 for the treatment of pulmonary emphysema.

For the applicants:

Cynthia Webb

Webb & Associates
Patent attorneys

A



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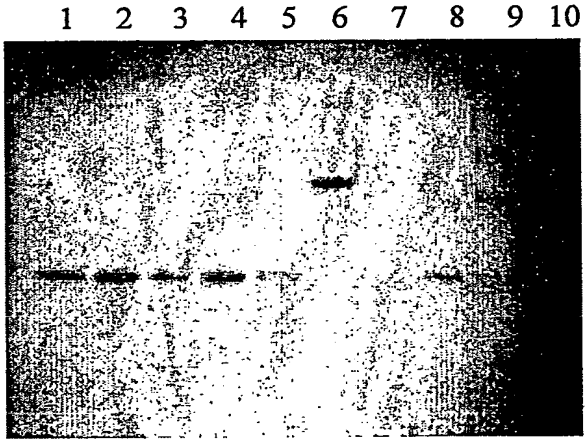

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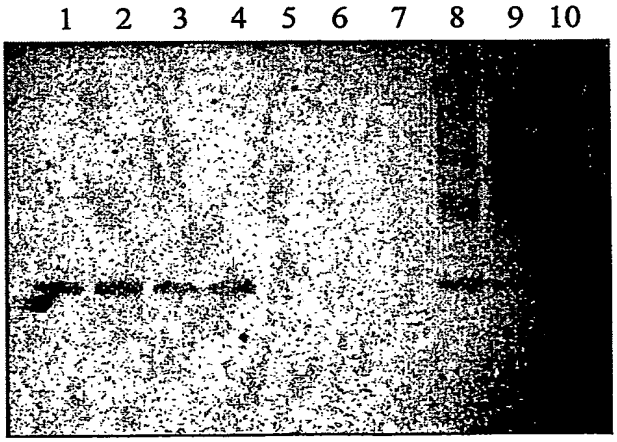
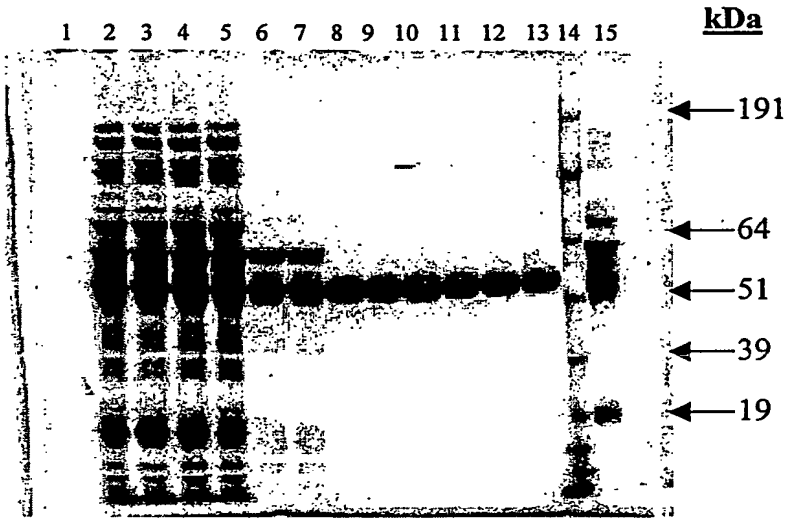


FIGURE 1

A



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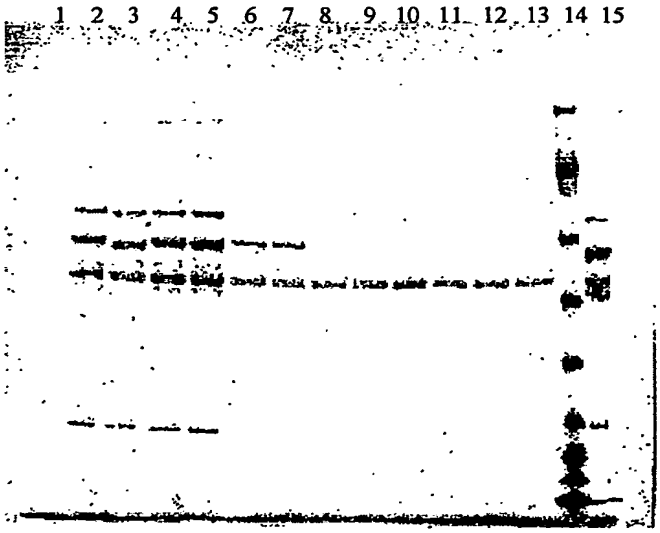

FIGURE 2

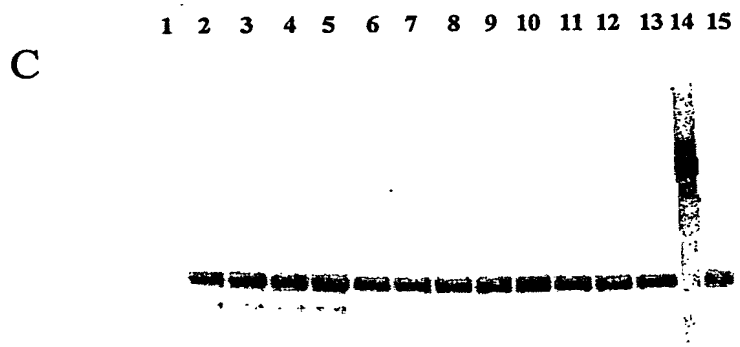

FIGURE 2